12. McDonald, J. C. & Whitesides, G. M. Poly(dimethylsiloxane) as a material for fabricating microfluidic devices. *Accounts Chem. Res.* **35,** 491–499 (2002).
13. Furlong, E. E. M., Profitt, D. & Scott, M. P. Automated sorting of live transgenic embryos. *Nature Biotechnol.* **19,** 153–156 (2001).
14. DiGregorio, P. J., Ubersax, J. A. & O'Farrell, P. H. Hypoxia and nitric oxide induce a rapid, reversible cell cycle arrest of the *Drosophila* syncytial divisions. *J. Biol. Chem.* **276,** 1930–1937 (2001).
15. Stasiek, J. A. & Kowalewski, T. A. Thermochromic liquid crystals applied for heat transfer research. *Opto-Electron. Rev.* **10,** 1–10 (2002).
16. Foe, V. E., Odell, G. M. & Edgar, B. A. in *The Development of Drosophila melanogaster* (eds Bate, M. & Martinez Arias, A.) (Cold Spring Harbor Press, New York, 1993).
17. Niemuth, J. & Wolf, R. Developmental asynchrony caused by steep temperature-gradients does not impair pattern-formation in the wasp, *Pimpla turionellae* L. *Roux's Arch. Dev. Biol.* **204,** 444–452 (1995).
18. Frasch, M. *et al.* Characterization and localization of the Even-skipped protein of *Drosophila*. *EMBO J.* **6,** 749–759 (1987).
19. Myasnikova, E. *et al.* Registration of the expression patterns of *Drosophila* segmentation genes by two independent methods. *Bioinformatics* **17,** 3–12 (2001).
20. Patel, N. H., Condron, B. G. & Zinn, K. Pair-rule expression patterns of Even-skipped are found in both short-germ and long-germ beetles. *Nature* **367,** 429–434 (1994).
21. Witkowski, F. X. *et al.* Spatiotemporal evolution of ventricular fibrillation. *Nature* **392,** 78–82 (1998).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements This work was supported by the Searle Scholars Program and was performed at the Chicago MRSEC microfluidic facility funded by the NSF. N.H.P. is an Investigator of the Howard Hughes Medical Institute. We thank J. B. Brokaw, C. A. Macrander and M. Giorgianni for preliminary experiments. We thank D. Bilder and I. Hariharan for discussions and comments on the manuscript.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to R.F.I. (r-ismagilov@uchicago.edu) and N.H.P. (nipam@uclink.berkeley.edu).

IKKα limits macrophage NF-κB activation and contributes to the resolution of inflammation

Toby Lawrence[1]*†, Magali Bebien[1]*, George Y. Liu[2], Victor Nizet[2] & Michael Karin[1]

[1]*Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology, and* [2]*Division of Paediatric Infectious Diseases, School of Medicine, University of California San Diego, 9500 Gilman Drive, California 92093, USA*

* These authors contributed equally to this work
† Present address: Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College, 1 Aspenlea Road, London W6 8LH, UK

Inflammation and innate immunity involve signalling pathways leading to the production of inflammatory mediators. Usually such responses are self-limiting, but aberrant resolution of inflammation results in chronic diseases[1]. Much attention has focused on pro-inflammatory signalling but little is known about the mechanisms that resolve inflammation. The IκB kinase (IKK) complex contains two catalytic subunits, IKKα and IKKβ, and controls the activation of NF-κB transcription factors, which play a pivotal role in inflammation[2]. Ample evidence indicates that IKKβ mediates NF-κB activation in response to pro-inflammatory cytokines and microbial products. IKKα regulates an alternative pathway important for lymphoid organogenesis[2], but the role of IKKα in inflammation is unknown. Here we describe a new role for IKKα in the negative regulation of macrophage activation and inflammation. IKKα contributes to suppression of NF-κB activity by accelerating both the turnover of the NF-κB subunits RelA and c-Rel, and their removal from pro-inflammatory gene promoters. Inactivation of IKKα in mice enhances inflammation and bacterial clearance. Hence, the two IKK catalytic subunits have evolved opposing but complimentary roles needed for the intricate control of inflammation and innate immunity.

NF-κB transcription factors are pivotal regulators of inflammation and immunity that control expression of important immunoregulatory genes[2,3]. NF-κB activation and activity are tightly controlled by a number of endogenous mechanisms that limit the excessive and prolonged production of pro-inflammatory mediators, which can cause tissue damage during the inflammatory response[3,4]. With the exception of autoregulated IκBα (inhibitor of NF-κB alpha) expression[4] and induction of the de-ubiquitinating enzyme A20 (ref. 5), the mechanisms that limit the duration and magnitude of NF-κB signalling are poorly understood. It is likely that IκBα and A20 are not the only physiologically relevant negative regulators of this central signalling module. The activation of NF-κB by pro-inflammatory stimuli depends on the classical IKK complex, composed of two catalytic subunits (IKKα and IKKβ), together with a regulatory subunit IKKγ/NEMO[3,6]. IKK activation is triggered by engagement of cytokine receptors as well as pattern recognition receptors. Gene disruption studies revealed that in addition to IKKγ/NEMO, which is necessary for activation of the classical IKK complex[7], it is IKKβ rather than IKKα that plays a more critical role in activating inflammation[6]. IKKα forms an alternative complex (without IKKβ and IKKγ)[8], the function of which is required for lymphoid organ development and B cell maturation[9]. This alternative signalling pathway is activated by certain members of the tumour necrosis factor (TNF) family, but not by pattern recognition receptors such as Toll-like receptor 4 (TLR4) (ref. 2). The function of IKKα within the classical IKK complex, however, is not entirely clear. Although a chromatin-modifying function for IKKα required for TNFα-mediated gene induction has been suggested[10,11], targeting of the *Ikkα* (also called *Chuk*) gene in mice does not support this proposal[2].

We investigated the role of IKKα in inflammation and innate immunity *in vivo*, using mice that express the inactivatable variant IKKα(AA) (ref. 9). $Ikk\alpha^{AA/AA}$ mice (which are homozygous for the mutant allele) and littermate controls were challenged systemically with the Gram-positive human pathogen group B *Streptococcus* (GBS)[12], and monitored for bacterial clearance and survival. Although $Ikk\alpha^{AA/AA}$ mice showed significantly decreased blood bacterial counts at 24 h (Fig. 1a), mortality was notably accelerated relative to wild-type animals (Fig. 1b). This paradoxical result suggested that $Ikk\alpha^{AA/AA}$ mice have an exacerbated inflammatory response to infection that enhances bacterial clearance but provokes septic shock. To pursue this hypothesis further, we assessed the local inflammatory response to bacterial infection in a non-lethal model. When $Ikk\alpha^{AA/AA}$ mice and littermate controls were inoculated intranasally with GBS, the mutants showed increased bacterial clearance, associated with increased neutrophil recruitment and local inflammation (Fig. 1c–e). Together, these studies indicate that IKKα is somehow involved in limiting the inflammatory response to Gram-positive infection.

We extended these studies to a model of Gram-negative septic shock by using the TLR4 agonist lipopolysaccharide (LPS) from *Escherichia coli*[13]. $Ikk\alpha^{AA/AA}$ mice showed increased susceptibility to LPS-induced septic shock (Fig. 2a). Real-time quantitative polymerase chain reaction (PCR) analysis of liver and lung RNA showed elevated expression of pro-inflammatory and anti-apoptotic NF-κB target genes, including macrophage inflammatory protein (MIP)-2, MIP-1α, interleukin (IL)-12p40, inhibitor of apoptosis protein (IAP)-2 and inducible nitric oxide synthase (iNOS) (Fig. 2b). Local LPS administration to the lung also resulted in elevated pulmonary leukocyte recruitment and pro-inflammatory cytokine production in $Ikk\alpha^{AA/AA}$ mice relative to wild-type littermates (see Supplementary Fig. 1). Neutrophil

recruitment in zymosan A-induced peritonitis (a TLR2-dependent stimulus[13,14]) was similarly exacerbated in *Ikkα*$^{AA/AA}$ mice, in association with increased chemokine and IL-12 release (see Supplementary Fig. 2). These findings raised the possibility that IKKα might serve to suppress or terminate activation of the classical NF-κB pathway during TLR signalling.

Further experiments demonstrated that the exacerbated inflammatory phenotype was intrinsic to the haematopoietic system. Lethally irradiated wild-type mice were reconstituted with bone marrow from *Ikkα*$^{AA/AA}$ mice or littermate controls. Mice receiving *Ikkα*$^{AA/AA}$ bone marrow were more susceptible to LPS-induced septic shock (Fig. 2c). Radiation chimaeras were also infected with GBS intranasally. Mice receiving *Ikkα*$^{AA/AA}$ bone marrow showed enhanced bacterial clearance (T. Lawrence, unpublished observations). These results are in contrast with the role of IKKα in lymphoid organogenesis, which is due to an intrinsic defect in lymphotoxin-β signalling in radiation-resistant stromal cells[15], and suggest a distinct function for IKKα in the resolution of inflammation in macrophages or other haematopoietic cells expressing TLRs.

Macrophages have a key role in innate immunity and inflammation[16]. Considering that enhanced inflammation in *Ikkα*$^{AA/AA}$ mice was intrinsic to the haematopoietic compartment, we examined the response of primary macrophages to bacteria and TLR agonists. Alveolar and peritoneal macrophages from *Ikkα*$^{AA/AA}$ mice show dramatically increased bactericidal activity against GBS *in vitro* (Fig. 3a). An important aspect of GBS evasion of the immune system is the induction of macrophage apoptosis[12], a response that is suppressed in similar models by IKKβ-driven NF-κB activation[17]. We found that *Ikkα*$^{AA/AA}$ macrophages were more resistant to GBS-induced apoptosis *in vitro* and *in vivo* compared with their wild-type counterparts (Fig. 3b). Real-time PCR analysis revealed increased induction of messenger RNAs for NF-κB-dependent anti-apoptotic and pro-inflammatory genes in GBS-infected *Ikkα*$^{AA/AA}$ macrophages (Fig. 3c). *In vitro*, *Ikkα*$^{AA/AA}$ macrophage resistance to GBS-induced cell death was associated with sustained iNOS expression and nitric oxide production (Supplementary Fig. 3), which is an important antimicrobial mechanism against GBS infection[18]. These effects of IKKα inactivation were mimicked by the addition of exogenous granulocyte–macrophage colony-stimulating factor (GM-CSF), a macrophage survival factor, to wild-type cells (Supplementary Fig. 3). We suggest that aberrant regulation of apoptotic pathways, leading to protracted survival of

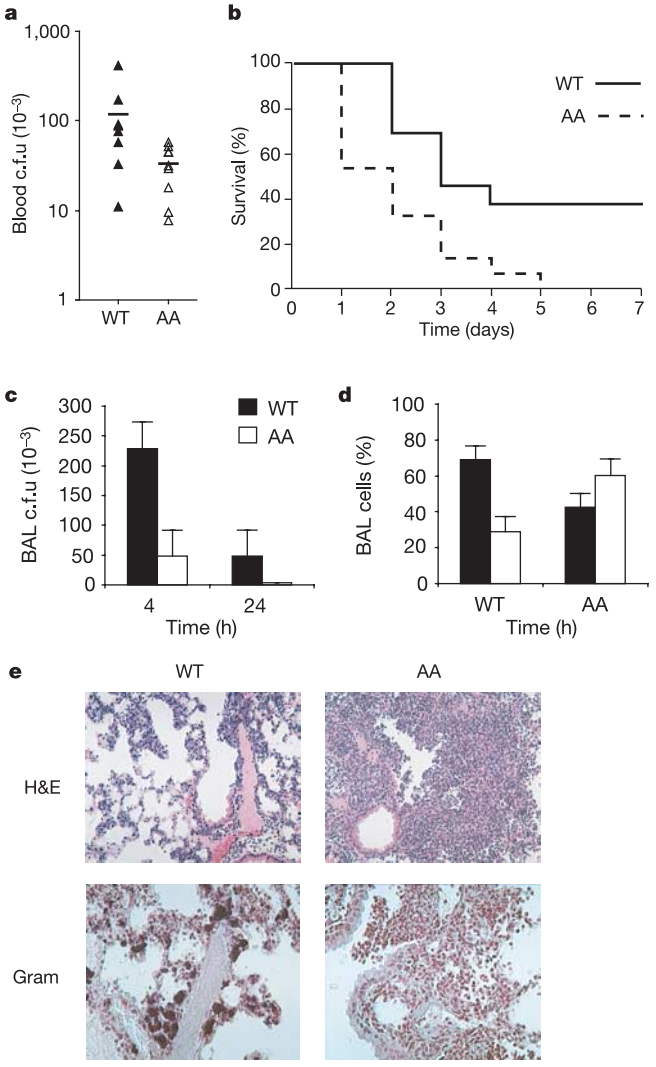

Figure 1 IKKα limits the inflammatory response to Gram-positive infection. **a**, GBS titres in blood were measured after intravenous inoculation with 5×10^6 c.f.u. bacteria. Data are represented as mean c.f.u. from individual wild-type (WT, closed triangles) and *Ikkα*$^{AA/AA}$ (AA, open triangles) mice, median is indicated by a solid bar. **b**, Kaplan–Meier survival plot of AA and WT littermates ($n = 11$). **c**, GBS-induced pneumonia in WT and AA mice, represented as bacterial titres in bronchial-alveolar lavage (BAL) ($n = 6$–8). **d**, Differential cell counts of bronchial-alveolar lavage, with data showing percentages of neutrophils (open bars) and macrophages (filled bars). **e**, Histopathology of GBS-induced pneumonia in WT and AA mice. Gram and haematoxylin-eosin staining (H&E) of lung tissue (original magnification × 400). Data in **c** and **d** show mean ± s.d. ($n = 6$–8).

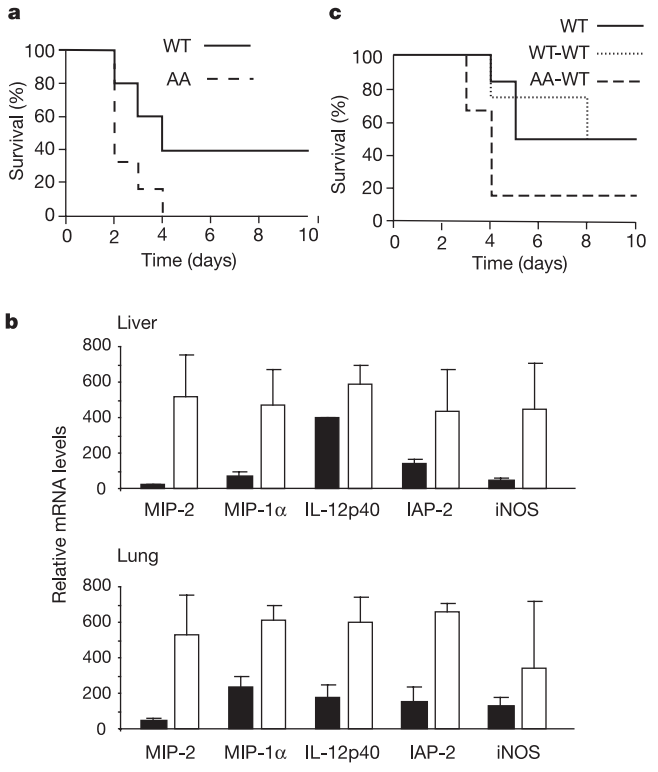

Figure 2 IKKα deficiency in haematopoietic cells increases the systemic inflammatory response to LPS. **a**, Kaplan–Meier survival plot for WT and AA mice ($n = 6$–8) after intraperitoneal injection of *E. coli* LPS. **b**, Lung and liver were harvested 24 h after LPS injection and RNA was isolated for real-time PCR analysis. Data are mean relative mRNA levels in WT (filled bars) and AA (open bars) mice, normalized to cyclophillin mRNA expression and shown as mean ± s.d. ($n = 3$–4). **c**, Kaplan–Meier survival plots for radiation chimaera mice. Wild-type mice were lethally irradiated and reconstituted with WT (WT-WT) or AA (WT-AA) bone marrow. After 8 weeks, mice were challenged with LPS.

activated macrophages, may in part explain the phenotype of enhanced inflammation and bactericidal function in $Ikk\alpha^{AA/AA}$ mice[12].

Further studies using real-time PCR showed increased pro-inflammatory and anti-apoptotic gene expression in $Ikk\alpha^{AA/AA}$ macrophages challenged with LPS *in vitro* (Fig. 4a). Elevated expression of such genes was confirmed by ribonuclease (RNase) protection assays (Fig. 4b), indicating that the major effect of the mutation was increased duration of NF-κB-dependent gene induction. Accordingly, the initial induction of NF-κB DNA-binding activity was not significantly elevated in LPS-stimulated $Ikk\alpha^{AA/AA}$ macrophages, but at later time points NF-κB DNA-binding activity was increased in the mutant cells relative to wild-type controls (Fig. 4c). Notably, increased nuclear levels of the NF-κB subunits RelA and c-Rel were detected at later time points in $Ikk\alpha^{AA/AA}$ relative to wild-type macrophages (Fig. 4c). Chromatin immunoprecipitation (ChIP) assays showed that the duration of RelA and c-Rel residence at the Bcl-2 family member A1 (Bfl-1),

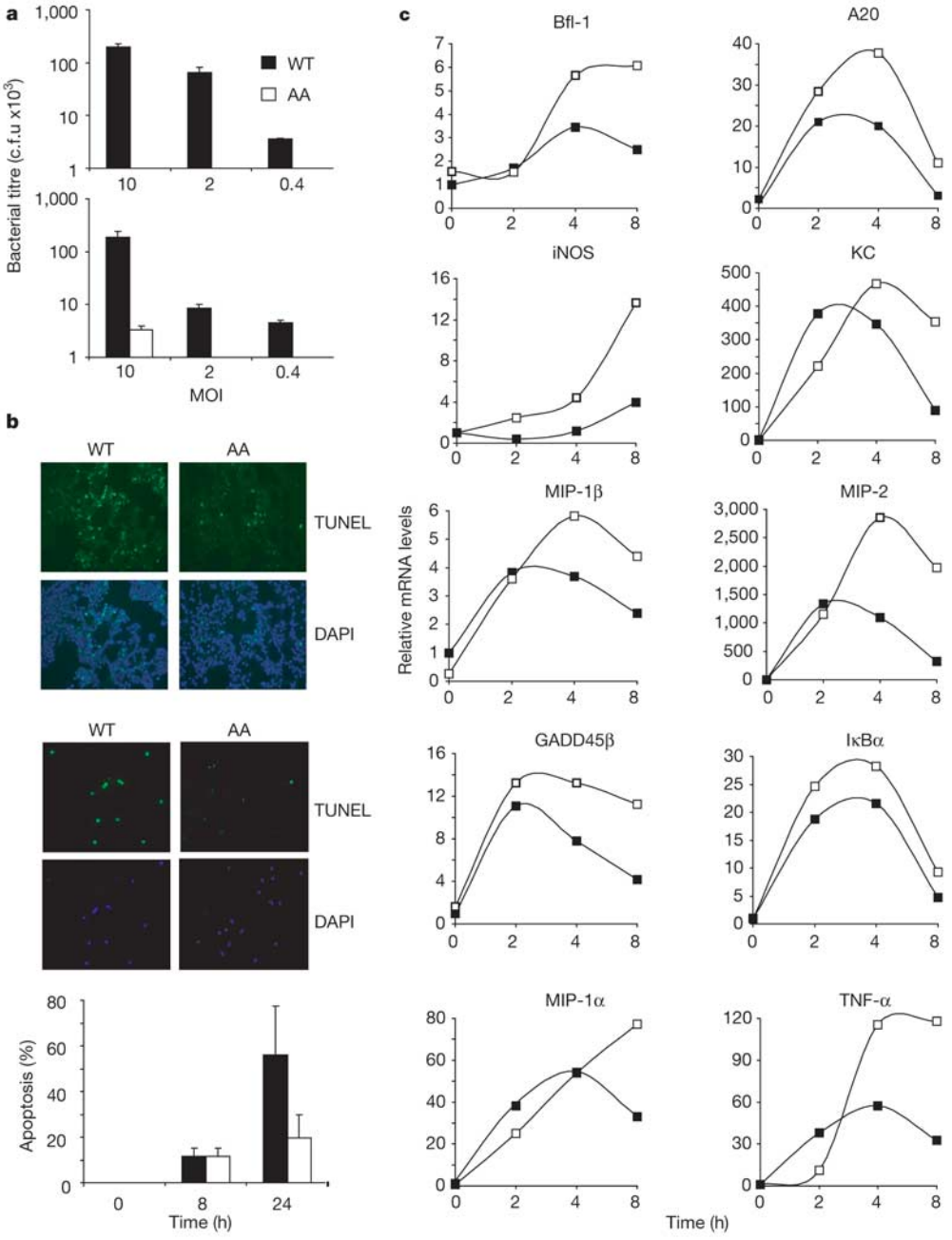

Figure 3 IKKα suppresses macrophage activation in response to GBS infection *in vitro*. **a**, Alveolar macrophages (top panel) and peritoneal macrophages (bottom panel) from WT and AA mice were infected with GBS, and intracellular titres of bacteria were determined 4 h later. Very low bacterial titres were recovered from AA macrophages. **b**, Top panel, lung tissue sections from WT and AA mice were examined by TUNEL assay after intranasal infection with GBS. Middle panel, TUNEL assay on peritoneal macrophages after infection with GBS at an MOI = 10:1. Bottom panel, quantification of apoptosis in GBS-infected macrophages (WT, filled bars; AA, open bars). Data in **a** and **b** show mean ± s.d. ($n = 3$). **c**, RNA was isolated from GBS-infected peritoneal macrophages, and gene expression was quantified by real-time PCR and normalized to the level of cyclophilin mRNA. WT, filled squares; AA, open squares. KC, mouse homologue of human melanoma growth stimulatory activity (MGSA); GADD45β, growth arrest DNA damage-inducible gene 45β.

IL-12p40 and iNOS gene promoters was extended in LPS-stimulated $Ikk\alpha^{AA/AA}$ macrophages relative to wild-type cells (Fig. 4d). Collectively, these results suggest that IKKα activation might be required for accelerated promoter clearance of RelA- or c-Rel-containing NF-κB complexes during resolution of the inflammatory response.

Despite the changes described above, the kinetics of IKKβ-dependent IκBα phosphorylation and degradation in LPS-stimulated macrophages were not affected by the $Ikk\alpha^{AA}$ mutation (Fig. 5a). The IKK complex is suggested to phosphorylate the carboxy-terminal activation domain of RelA[19], but the physiological function of this phosphorylation and the IKK subunit that mediates it are unknown. In contrast to the normal levels of IκBα kinase activity, $Ikk\alpha^{AA/AA}$ macrophages showed reduced activation of an IKKγ-associated RelA kinase (Fig. 5b). The amino acid target for this kinase activity is likely to be serine 536, because a variant of RelA in which this serine was replaced with alanine was phosphorylated equally well by IKK complexes from wild-type or $Ikk\alpha^{AA/AA}$ macrophages (see Supplementary Fig. 4a). In similar experiments, IKK complexes from LPS-stimulated macrophages did not phosphorylate an amino-terminal fragment of RelA (amino acids 1–305) (data not shown). Immunoblot analysis with phospho-specific antibodies that recognize RelA phosphorylated at either S536 or S276 showed diminished S536 phosphorylation in LPS-stimulated $Ikk\alpha^{AA/AA}$ macrophages, but S276 phosphorylation remained intact (Fig. 5c). c-Rel is also subjected to C-terminal phosphorylation[20,21], and the C-terminal kinase activity of c-Rel was also decreased in IKK complexes from $Ikk\alpha^{AA/AA}$ macrophages, provided that the c-Rel fragment used in these assays was restricted to residues 422–540; phosphorylation of a longer c-Rel substrate encompassing residues 422–588 was not as extensively reduced (see Supplementary Fig. 4b).

Promoter clearance of sequence-specific transcription factors is suggested to be mediated by ubiquitin-dependent proteolysis[22–24], which is closely linked to inducible phosphorylation and ubiquitination of transcription factor trans-activation domains[22]. We therefore examined whether IKKα was involved in Rel protein turnover during the course of macrophage activation. Previous studies have demonstrated ubiquitination and proteasomal degradation of both RelA and c-Rel[21,24–26], and it has been suggested that RelA ubiquitination leads to recruitment of proteasome components to target gene promoters and to RelA proteolysis[24]. This mechanism was proposed to be involved in the termination of NF-κB activation.

We stimulated primary macrophages with LPS and followed the kinetics of NF-κB activation in the absence or presence of a proteasome inhibitor. Proteasome inhibition prolonged the LPS-induced DNA-binding activity of NF-κB and increased the nuclear abundance of RelA and c-Rel (see Supplementary Fig. 5). We performed pulse-chase experiments using ^{35}S-labelled amino

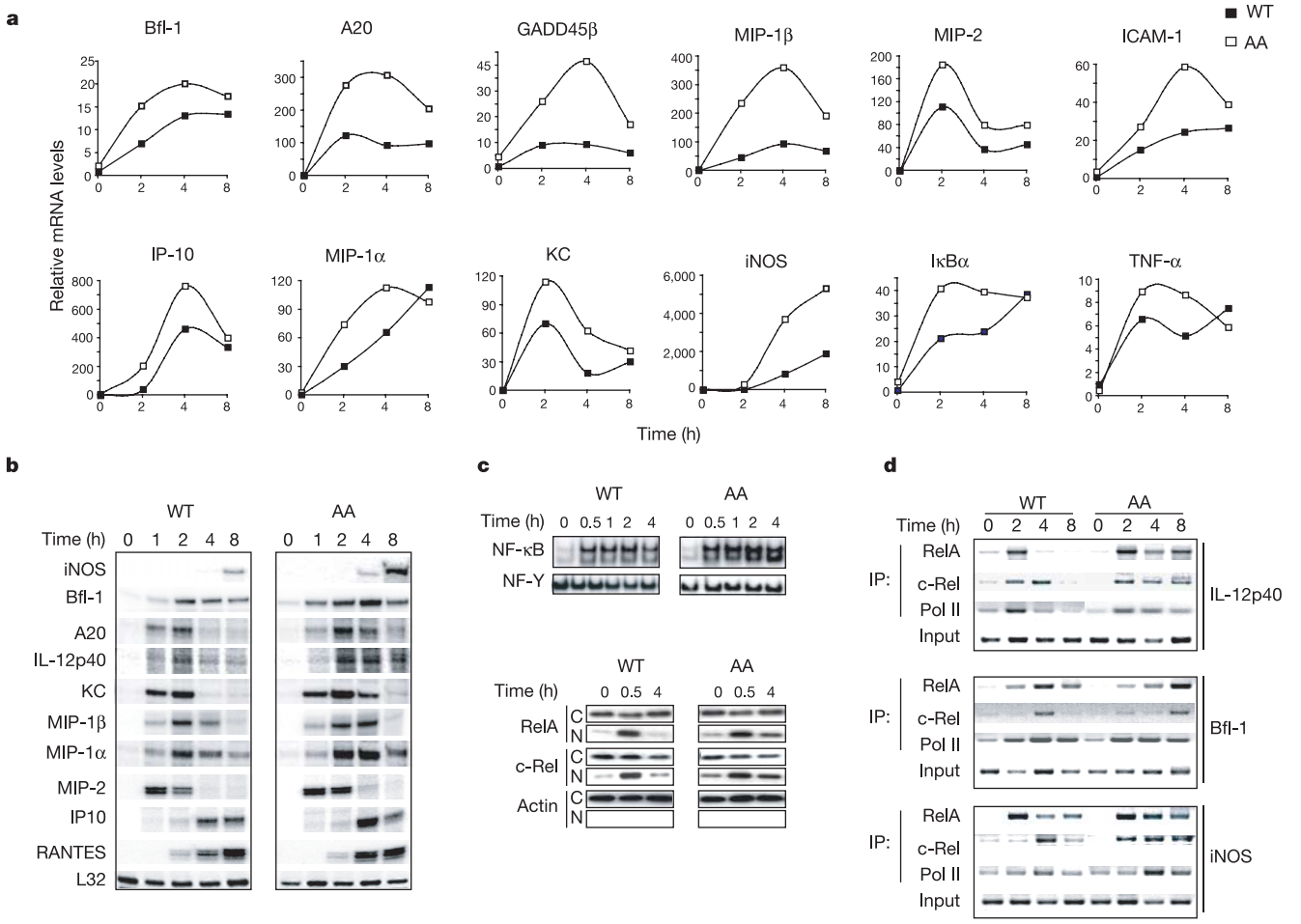

Figure 4 IKKα negatively regulates RelA and c-Rel nuclear accumulation and NF-κB target gene expression in LPS-stimulated macrophages. **a**, **b**, Total RNA was isolated from bone-marrow-derived macrophages at the indicated time points for real-time PCR analysis (**a**) and RNase protection assays (**b**). **c**, Kinetics of NF-κB DNA-binding activity (top panel) and nuclear translocation of RelA and c-Rel proteins (bottom panel) in WT and AA macrophages after LPS stimulation. **d**, Chromatin immunoprecipitation assays. Recruitment of RelA, c-Rel and RNA polymerase II to the Bfl-1, iNOS and IL-12p40 promoters was assessed by immunoprecipitation and PCR amplification of promoter sequences.

acids to track the turnover of Rel proteins in LPS-stimulated wild-type and *Ikkα*$^{AA/AA}$ macrophages. These experiments revealed a considerably increased half-life ($t_{1/2}$) for both RelA and c-Rel in LPS-stimulated *Ikkα*$^{AA/AA}$ macrophages, whereas the turnover of RelB (an NF-κB subunit associated with anti-inflammatory activity[27]) was only modestly affected (Fig. 5d). Transfection studies revealed that replacing S536 of RelA with alanine abrogated LPS-induced turnover, but mutation of S276 had no effect (Fig. 5e). Furthermore, LPS-induced RelA turnover was abrogated in *Ikkα*$^{-/-}$ cells (Fig. 5e). Thus, IKKα activation accelerates RelA and c-Rel proteolysis and promotes their clearance from target gene promoters through their C-terminal phosphorylation. This mechanism may explain macrophage hyperactivity and increased inflammation in *Ikkα*$^{AA/AA}$ mice.

The studies described above reveal opposing yet complimentary roles for IKKα and IKKβ in the control of inflammation and innate immunity. We demonstrate a previously unknown function for IKKα in negative regulation of macrophage activation. This function might be mediated through IKKα-dependent phosphorylation of RelA and c-Rel, which results in accelerated turnover of these NF-κB subunits, thereby facilitating their removal from target gene promoters and terminating NF-κB-mediated gene induction. This model is supported by the protracted induction of a number of NF-κB target genes in *Ikkα*$^{AA/AA}$ macrophages and the extended retention time of RelA and c-Rel at their promoters. In addition, the half-life of RelA and c-Rel is significantly increased in *Ikkα*$^{AA/AA}$ macrophages, but the half-life of RelB associated with the termination of NF-κB-mediated gene induction[28], is only modestly affected. The connection between IKKα-dependent RelA C-terminal phosphorylation and stimulus-induced turnover is confirmed in transfection experiments that reveal S536 as a target for IKKα-dependent phosphorylation, showing that an S536A mutation abrogates LPS-induced RelA turnover. It is therefore evident that IKKα-dependent functions are associated with the termination of NF-κB-dependent transcription of pro-inflammatory genes. Impaired IKKα activation removes a 'brake' on macrophage activation and increases expression of pro-inflammatory genes, leading to elevated local inflammation, enhanced macrophage resistance to pathogen-induced apoptosis, and increased innate immunity to bacterial pathogens.

It would appear that the IKK complex has evolved to promote rapid but transient nuclear localization of Rel transcription factors in response to pro-inflammatory stimuli. Given that the first step in NF-κB activation, which depends on IKKβ, involves irreversible IκBα degradation[3], a second step is necessary to ensure the transient nature of this signalling response. Part of this depends on inducible IκBα expression[4,29], but that may not be sufficient. The IKKα-dependent step may have evolved to ensure the rapid turnover of pro-inflammatory RelA- and c-Rel-containing dimers and their replacement with anti-inflammatory RelB-containing dimers[28]. The cytoplasmic localization of the IKK complex would imply that C-terminal phosphorylation of RelA and c-Rel occurs in this compartment, while still associated with IκBs; this might involve the newly discovered ELKS subunit[30]. However, the phosphorylated proteins are probably degraded mostly in the nucleus, as suggested by cell fractionation experiments (Fig. 4c). Selective inhibition of

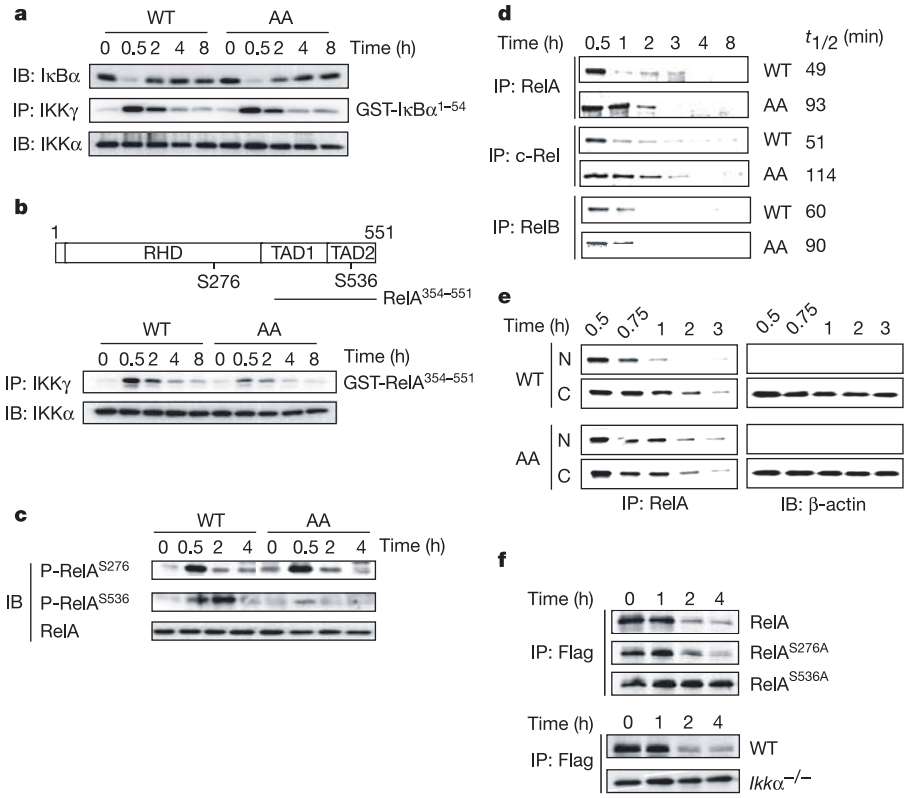

Figure 5 IKKα mediates C-terminal RelA phosphorylation and promotes its stimulus-induced turnover. **a**, **b**, Immune complex kinase assays performed with GST-IκBα(1–54) (**a**) and GST-RelA(354–551) (**b**). **c**, Immunoblot (IB) analysis of RelA phosphorylation. **d**, Pulse-chase analysis of RelA, c-Rel and RelB in LPS-stimulated WT and AA macrophages. Half-life ($t_{1/2}$) was calculated from a semi-log plot of three independent experiments. **e**, Pulse-chase analysis of nuclear (N) and cytoplasmic (C) fractions of RelA in LPS-stimulated WT and AA macrophages. **f**, Top panel, transient transfection with Flag-RelA, Flag-RelA(S276A) or Flag-RelA(S536A) mutants. Turnover of immunoprecipitated Flag-tagged proteins was assessed as above. Bottom panel, WT and *Ikkα* $^{-/-}$ murine embryonic fibroblast cells were transfected with Flag-RelA and protein turnover was assessed as above.

IKKα activation, although having no effect on IκBα degradation, has the unusual effect of enhancing innate immunity by preventing RelA and c-Rel turnover, thereby causing protracted NF-κB activation. This discovery may present new therapeutic opportunities for IKKα inhibitors in the treatment of complicated infections involving antibiotic resistance or compromised host immunity. □

Methods

Bacterial infections

The clinical GBS isolate, NCTC10/84 (serotype V)[12] was grown in Todd Hewitt broth (THB) without agitation at 37 °C to an absorbance at 600 nm of 0.4, equivalent to 1×10^8 c.f.u. per ml. Bacteria collected by centrifugation were washed with sterile PBS. Mice were inoculated via the tail vein with 5×10^7 c.f.u. GBS in 0.3 ml PBS. Blood was collected from mice after 24 h by retro-orbital bleed, serial dilutions were plated on Todd Hewitt agar (THA) in triplicate and colonies were counted. Mice were also inoculated intranasally with 3×10^7 c.f.u. NCTC in 30 μl PBS. These mice were killed at the indicated time points, and their tracheas were cannulated for bronchial-alveolar lavage with 3 aliquots of 0.8 ml ice-cold PBS. Serial dilutions of bronchial-alveolar lavage fluid were plated on THA in triplicate and c.f.u. counts determined. Lung tissue was also prepared for routine histological analysis.

LPS-induced septic shock

Mice were challenged by intraperitoneal injection of 25 mg kg^{-1} LPS (from *E. coli* serotype B5:055, Sigma) in pyrogen-free PBS. Chimaeric mice were generated using bone marrow from *Ikkα*$^{AA/AA}$ and wild-type littermate controls. Bone marrow cells were isolated in Hank's balanced salt solution (HBSS) and within 6 h, 5×10^6 cells in 0.3 ml were injected into the tail vein of 8-week-old lethally irradiated wild-type hosts[9]. From 2 days before injection, host mice were housed under sterile conditions, using autoclaved cages, food, and water containing 25 mg l^{-1} neomycin sulphate and 13 mg l^{-1} polymyxin B sulphate.

Macrophage isolation and stimulation

Bone marrow-derived macrophages (BMDMs) were generated as described[17]. Peritoneal macrophages were elicited by intraperitoneal injection of 3 ml of 3% thioglycollate in distilled water. After 3 days, cells were harvested and plated in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U ml^{-1} penicillin and streptomycin, and 2 mM glutamine. BMDMs were stimulated with 100 ng ml^{-1} LPS for 30 min, after which the cells were washed and incubated in LPS-free media. For infection experiments, peritoneal macrophages were washed with antibiotic-free media and incubated with the indicated MOI (multiplicity of infection) of GBS, prepared as described above. Culture plates were centrifuged to bring bacteria into contact with macrophages and incubated for 2 h, after which cells were washed with media supplemented with 20 μg ml^{-1} gentamicin and incubated for a further 2 h. Macrophages were then washed and intracellular bacteria were released with 0.02% Triton X-100. Surviving bacteria were counted by plating serial dilutions in triplicate on THA. Apoptosis was measured by TUNEL assay using the ApoAlert kit (BD Bioscience). Nuclear morphology was assessed by DAPI counter-stain.

Gene expression analysis and chromatin immunoprecipitation assays

Total cellular RNA was isolated using TRIzol (Invitrogen) and analysed by real-time PCR with SyBr Green (PE Biosystems 5700 thermocycler) or RNase protection assay. Primer sequences are available upon request. For real-time PCR analyses, all values were normalized to the level of cyclophilin mRNA. For RNase protection assays, total RNA was hybridized with RNA probes using a Riboquant Multiprobe RPA System (BD Bioscience), following manufacturer's instructions. ChIP assays were performed as described[24] using either anti-RelA (C-20), anti-c-Rel (C) or anti-Pol II (N-19) polyclonal antibodies (Santa Cruz) for immunoprecipitation. Sequences of promoter-specific primers and a detailed experimental protocol are available upon request.

Kinase assay, immunoblotting and pulse-chase

Whole-cell lysates were prepared and IKK kinase activity was measured after immunoprecipitation with anti-IKKγ (764) antibody (BD Pharmingen) as described[9], using the following substrates: GST-IκBα(1–54), GST-RelA(354–551), GST-RelA(354–551; S536A), GST-c-Rel(422–588), GST-c-Rel(422–540). IKK recovery was determined by immunoblotting with anti-IKKα (M280) antibody (Santa Cruz). Immunoblotting was performed on gel-separated whole-cell lysates or on nuclear and cytoplasmic extracts[9]. For pulse-chase analyses, macrophages were labelled for 1 h with 100 μCi ml^{-1} [35S]-methionine and [^{35}S]-cysteine (ICN Biomedicals). The cells were washed and chased for the indicated time with fresh medium containing unlabelled amino acids. Cells were collected, cell lysates were pre-cleared using protein G-Sepharose, and proteins were immunoprecipitated with anti-RelA, anti-RelB, anti-cRel or anti-Flag antibodies. Immune complexes were resolved by SDS–PAGE and the gels were dried and autoradiographed. Radiolabelled protein bands were quantified using a phosphorimager.

Transfection

Murine embryonic fibroblast (MEF) cells from wild-type or *Ikkα*$^{-/-}$ mice were cultured in 60-mm dishes and transfected with plasmid DNAs encoding Flag-RelA, Flag-RelA(S276A), or Flag-RelA(S536A) using LipofectAMINE Plus (Invitrogen, Gibco BRL) following the manufacturer's instructions.

Received 15 October 2004; accepted 17 February 2005; doi:10.1038/nature03491.

1. Lawrence, T., Willoughby, D. A. & Gilroy, D. W. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nature Rev. Immunol.* **2,** 787–795 (2002).
2. Bonizzi, G. & Karin, M. The two NF-κB activation pathways and their role in innate and adaptive immunity. *Trends Immunol.* **25,** 280–288 (2004).
3. Karin, M. & Ben-Neriah, Y. Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annu. Rev. Immunol.* **18,** 621–663 (2000).
4. Hoffmann, A., Levchenko, A., Scott, M. L. & Baltimore, D. The IκB-NF-κB signaling module: temporal control and selective gene activation. *Science* **298,** 1241–1245 (2002).
5. Wertz, I. E. *et al.* De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signalling. *Nature* **430,** 694–699 (2004).
6. Ghosh, S. & Karin, M. Missing pieces in the NF-κB puzzle. *Cell* **109** (suppl.), S81–S96 (2002).
7. Makris, C. *et al.* Female mice heterozygous for IKKγ/NEMO deficiencies develop a dermatopathy similar to the human X-linked disorder incontinentia pigmenti. *Mol. Cell* **5,** 969–979 (2000).
8. Dejardin, E. *et al.* The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-κB pathways. *Immunity* **17,** 525–535 (2002).
9. Senftleben, U. *et al.* Activation by IKKα of a second, evolutionary conserved, NF-κ B signaling pathway. *Science* **293,** 1495–1499 (2001).
10. Anest, V. *et al.* A nucleosomal function for IκB kinase-α in NF-κB-dependent gene expression. *Nature* **423,** 659–663 (2003).
11. Yamamoto, Y., Verma, U. N., Prajapati, S., Kwak, Y. T. & Gaynor, R. B. Histone H3 phosphorylation by IKK-α is critical for cytokine-induced gene expression. *Nature* **423,** 655–659 (2003).
12. Liu, G. Y. *et al.* Sword and shield: Linked group B streptococcal β-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. *Proc. Natl Acad. Sci. USA* **101,** 14491–14496 (2004).
13. Takeuchi, O. *et al.* Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11,** 443–451 (1999).
14. Underhill, D. M. *et al.* The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* **401,** 811–815 (1999).
15. Bonizzi, G. *et al.* Activation of IKKα target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. *EMBO J.* **23,** 4202–4210 (2004).
16. Gordon, S. Pattern recognition receptors: doubling up for the innate immune response. *Cell* **111,** 927–930 (2002).
17. Park, J. M., Greten, F. R., Li, Z. W. & Karin, M. Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. *Science* **297,** 2048–2051 (2002).
18. Puliti, M., von Hunolstein, C., Bistoni, F., Orefici, G. & Tissi, L. Inhibition of nitric oxide synthase exacerbates group B streptococcus sepsis and arthritis in mice. *Infect. Immun.* **72,** 4891–4894 (2004).
19. Sakurai, H. *et al.* Tumor necrosis factor-alpha-induced IKK phosphorylation of NF-κB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. *J. Biol. Chem.* **278,** 36916–36923 (2003).
20. Martin, A. G. & Fresno, M. Tumor necrosis factor-alpha activation of NF-κB requires the phosphorylation of Ser-471 in the transactivation domain of c-Rel. *J. Biol. Chem.* **275,** 24383–24391 (2000).
21. Fognani, C., Rondi, R., Romano, A. & Blasi, F. cRel-TD kinase: a serine/threonine kinase binding *in vivo* and *in vitro* c-Rel and phosphorylating its transactivation domain. *Oncogene* **19,** 2224–2232 (2000).
22. Salghetti, S. E., Muratani, M., Wijnen, H., Futcher, B. & Tansey, W. P. Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. *Proc. Natl Acad. Sci. USA* **97,** 3118–3123 (2000).
23. Perissi, V., Aggarwal, A., Glass, C. K., Rose, D. W. & Rosenfeld, M. G. A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. *Cell* **116,** 511–526 (2004).
24. Saccani, S., Marazzi, I., Beg, A. A. & Natoli, G. Degradation of promoter-bound p65/RelA is essential for the prompt termination of the nuclear factor κB response. *J. Exp. Med.* **200,** 107–113 (2004).
25. Ryo, A. *et al.* Regulation of NF-κB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol. Cell* **12,** 1413–1426 (2003).
26. Chen, E. *et al.* Degradation of proto-oncoprotein c-Rel by the ubiquitin-proteasome pathway. *J. Biol. Chem.* **273,** 35201–35207 (1998).
27. Weih, F. *et al.* Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-κB/Rel family. *Cell* **80,** 331–340 (1995).
28. Saccani, S., Pantano, S. & Natoli, G. Modulation of NF-κB activity by exchange of dimers. *Mol. Cell* **11,** 1563–1574 (2003).
29. Sun, S. C., Ganchi, P. A., Ballard, D. W. & Greene, W. C. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. *Science* **259,** 1912–1915 (1993).
30. Ducut Sigala, J. L. *et al.* Activation of transcription factor NF-κB requires ELKS, an IκB kinase regulatory subunit. *Science* **304,** 1963–1967 (2004).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank H. Sakurai, H. Kamata, and G. Fresno for plasmid constructs, and J.M. Park and A. Hoffmann for discussions and advice. T.L. is the recipient of a Wellcome Trust International Research Fellowship. V.N. is an Edward J. Mallinckrodt, Jr Scholar. Research was supported by NIH grants to M.K., who is the Frank and Else Schilling American Cancer Society Research Professor.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to T.L. (t.lawrence@imperial.ac.uk).